Identity and Structural Organization of a Microbial Community in a Model Drinking Water Distribution System

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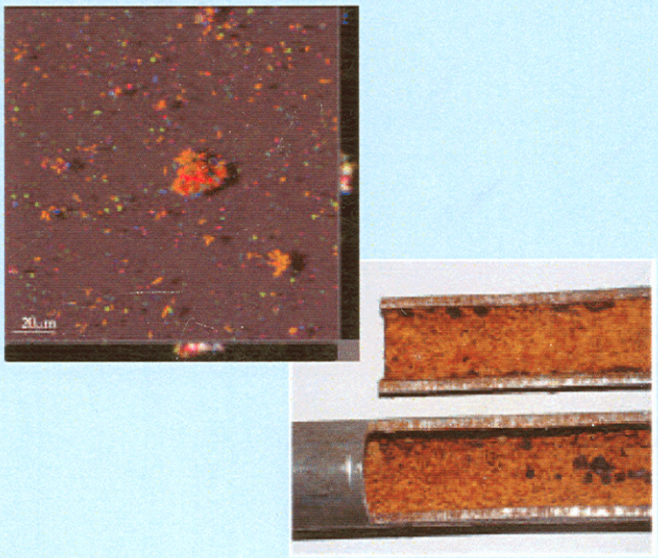

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Index

Paper 1. Martiny, A.C., Nielsen, A.T., Arvin, E., Molin, S., and H.-J. Albrechtsen. 2002. *In situ* examination of microbial populations in a model drinking water distribution system. Wat. Sci. Tech.: Wat. Suppl., 2, 283-288.

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Paper 3. Martiny, A.C., Albrechtsen, H.-J., Arvin, E., and S. Molin. 2003. A comparison between the culturable and total bacterial population in a model drinking water distribution system – detection of an unrecognized diversity. *In prep.*

Paper 4. Martiny, A.C., Jørgensen, T.M., Albrechtsen, H.-J., Arvin, E., and S. Molin. 2003. Long-term succession in structure and diversity of a biofilm formed in a model drinking water distribution system. Submitted to Applied and Environmental Microbiology.

Acknowledgement

My three-year Ph.D. study was carried out as a collaboration between the two departments Biocentrum-DTU (BiC) and Environment and Resources (E&R) at the Technical University of Denmark, with Søren Molin as supervisor, and Hans-Jørgen Albrechtsen and Erik Arvin as co-advisors.

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Summary

The aim of the present thesis is to determine the identity and structural organization of the indigenous bacteria present in a model drinking water distribution system. The study shows how unrecognized bacterial species in drinking water proliferate in two separate ecological compartments, in a biofilm on the pipe surface and free-swimming in the bulk water.

Initially, the development of a biofilm in a drinking water distribution system was analyzed with a simplified flow-cell system. The flow-cell set-up enabled a direct microscopic analysis of the biofilm. Microcolonies consisting of a mixed community including α- and â-Proteobacteria were seen with fluorescent in situ hybridization. Furthermore, a variety of protozoans were present in the system, and some were attached to the microcolonies.

A phylogenetic and physiological examination of isolates from two non-chlorinated drinking water distribution systems showed, that bacteria from the biofilm were on average able to utilize a higher number of substrates than strains from the bulk water. Despite differences in taxonomic affiliation of the strains in the two analyzed systems, a parallel distribution of genetic and physiological diversity in the biofilm and bulk water was observed.

However, in other environments, abundant bacteria have solely been detected using cultivation independent techniques. Using cloning and sequencing of 16S rRNA fragments, it was demonstrated that bacteria from at least 12 phyla were present in the Danish model distribution system, including some of which never have been detected in drinking water. A bacterium affiliated to a nitrite oxidizer, *Nitrospira*, encompassed 39% of the bulk water and 25% of the biofilm community. The close affiliation to *Nitrospira* suggested, that a large part of the population had an autotrophic metabolism. Bacteria affiliated to *Acidobacterium* and *Planctomycetes* were found in densities of up to 15%.

An analysis of the community composition using terminal restriction fragment length polymorphism showed a correlation between the population profile and age of biofilm, separating the samples into a young (1 – 94 days) and an old biofilm (571 – 1093 days), whereas limited spatial variation in the biofilm was observed. A more detailed analysis of 16S rRNA fragments demonstrated a unimodel relationship between the age and richness of the biofilm. Initially, a wide variety of cells were recruited from the bulk water colonizing the pipe surface and resulted in a species richness comparable to the water phase. This event was followed by growth of another bacterium from the phylum *Nitrospirae*, reaching 78% of the community by day 256. Moreover, the bloom of this specie resulted in a drop in the relative richness. The biofilm entered a stable population from 500 days and on-wards, that was characterized by a higher diversity of bacteria.

Visualization and subsequent quantification showed how the biofilm developed from an initial attachment of single cells, followed by formation of independent microcolonies reaching 30 µm in thickness, and finally to a looser structure with an average thickness of 14 µm and covering 76% of the surface. The combination of different techniques illustrated the successional formation of a biofilm during a 3-year period in this model drinking water distribution system.

A cluster analysis divided the young and old biofilm, and the bulk water communities into three separate groups. A detailed comparison between the communities in the biofilm and bulk water showed that certain species were solely

found in microhabitat, whereas other species were present in both the biofilm and adjacent water phase. Combined with the observed physiological difference between bacteria from the biofilm and bulk water, it appeared that many species had a primary habitat in either the biofilm or bulk water but that a dynamic exchange occurred between the communities.

Resumé pådansk

Formålet med dette studie er at undersøge identiteten og den strukturelle organisation af naturligt forekomne bakterier i et model-drikkevandsforsyningssystem. Studiet viser, hvordan ikke tidligere detekterede arter i drikkevand lever i to separate økologiske nicher, i en biofilm og fritsvømmende i vandfasen.

Et indledende studie undersøgte udviklingen af en biofilm i et simplificeret flow-celle system. Dette system muliggjorde en direkte mikroskopianalyse af biofilmdannelsen. Mikrokolonierne bestod af en blanding af α- og â-Proteobacteria visualiseret med *in situ* hybridisering. Forskellige protozoer, heraf nogle i forbindelse med mikrokolonierne, blev ligeledes observeret i biofilmen.

En fylogenetisk og fysiologisk analyse af isolater fra to ikke-klorinerede vandforsyningssystemer viste, at bakterier fra overfladen kunne udnytte et højere antal substrater i forhold til bakterier fra vandfasen. En parallel genetisk og fysiologisk fordeling blev observeret i de to vandforsyningssystemer, på trods af forskelle i den

I flere andre miljøer har man kun fundet dominerende bakterielle arter uafhængigt af dyrkning. I det danske modelsystem blev bakterier tilhørende 12 forskellige fylum påvist, v.h.a. kloning og sekventering, herunder arter som aldrig tidligere var fundet i drikkevand. En bakterie i familie med en nitrite-oxiderende art, *Nitrospira*, udgjorde 39 % af bakterierne i vandfasen og 25 % af biofilmen. Den tætte *Nitrospira* antydede, at en stor del af den mikrobielle population havde en autotrof metabolisme. Bakterier beslægtet til *Acidobacterium* og *Planctomycetes* udgjorde op til 15 % af den samlede population.

"Terminal restriction fragment length polymorphism" viste en korrelation mellem den mikrobielle populations sammensætning og alderen af biofilmen. Analysen delte prøverne i en ung (1 – 94 dage) og en gammel (571 – 1093 dage) biofilm, hvorimod en begrænset afstandsmæssig variation sås. En mere detaljeret analyse af 16S rRNA fragmenter demonstrede en u-formet sammenhæng mellem alder og artsrigdom i biofilmen. En række forskellige arter rekrutteret fra vandfasen startede med at kolonisere overfladen, hvilket resulterede i en artsrigdom på højde med vandfasens. Denne kolonisering blev efterfulgt af vækst af en stamme fra *Nitrospirae*, som dermed udgjorde 78 % af populationen ved dag 256. Opblomstringen af denne art resulterede i et fald i den relative artsrigdom. Biofilmen udgjorde et stabilt samfund efter cirka 500 dage karakteriseret af en højere bakteriel diversitet.

Visualiseringen af biofilmen og den efterfølgende kvantificering viste hvordan biofilmen udviklede sig fra at bestå af enkelte celler spredt ud på overfladen, efterfulgt af dannelsen af op til 30 µm tykke individuelle mikrokolonier, til afslutningsvis at bestå af en løsere struktur 14 µm høj og dækkende 76 % af overfladen i gennemsnit. Kombinationen af forskellige teknikker illustrerede over en treårig periode den successionelle dannelse af en biofilm i dette model-drikkevandssystem.

En "clusteranalyse" baseret på de tilstedeværende arter delte den unge biofilm, den gamle biofilm og vandfasen i tre grupper. En detaljeret sammenligning viste, at visse bakterier kun fandtes i enten vandet eller biofilmen, hvorimod andre arter fandtes i begge miljøer. Kombineret med forskellene i substratforbrug mellem vandfasen og biofilmen, foreslog analysen at mange arter har et foretrukken miljø men en dynamisk udveksling mellem de to populationer forekommer.

Introduction

Foreword

It is becoming clear, that despite our eradication of many pathogenic strains in potable water, other unwanted and potentially harmful bacteria are being recognized as present in the water supply systems in the absence of any clear correlation to a contamination event. When investigating drinking water distribution system, the primary focus has traditionally been the direct estimation of the fate and survival of pathogenic bacteria. In the first part of the introduction, I want to try to demonstrate that the fate of pathogens in the supply system is a result of a complex interaction between the cell and the local environment, and as an effect of this, the indigenous microbial population. The next part is a discussion of different techniques for examining bacteria in natural communities, since the characterization of the indigenous bacterial population partly is influenced by the method used for monitoring. This is followed by a description of analytical tools for deducting patterns in the distribution of species derived from an examination of complex microbial communities. The next part lists previously identified species in drinking water, including pathogenic and non-pathogenic bacteria, and their ecological role in water supply systems is discussed. In contrast to free-swimming, biofilms creates unique ecological opportunities for bacterial proliferation and survival. These niche disparities can result in a differentiation between biofilm and bulk water communities, which is discussed in last part of the introduction. The introduction is followed by four papers that represent the outcome of the research during my three-year Ph.D. project at the Molecular Microbial Ecology Group and Environment & Resources at DTU.

List of publications:

Martiny, A.C., Albrechtsen, H.-J., Arvin, E., and S. Molin. 2001. Application and strategy for identifying and monitoring bacterial fouling. *In* Månsson, L. (ed.), Proceedings from the 32nd R^3 symposium, Stockholm, Sweden.

Martiny, A.C., Nielsen, A.T., Arvin, E., Molin, S., and H.-J. Albrechtsen. 2002. *In situ* examination of microbial populations in a model drinking water distribution system. Wat. Sci. Tech.: Wat. Suppl., 2, 283-288.

Boe-Hansen, R., **Martiny, A.C.,** Arvin, E., and H.-J. Albrechtsen. 2003.Monitoring biofilm formation and activity in drinking water distribution networks under oligotrophic conditions. Wat. Sci. Tech. 47 (5) 91 – 97.

Martiny, A.C., Conradi, B., Albrechtsen, H.-J., Arvin, E., Szewzyk, U. Manz, W., and S. Molin. 2003. A Phylogenetic and Physiological Examination of Attached and Planktonic Bacteria Isolated from Two Model Drinking Water Distribution Systems. Submitted to Water Research.

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Holm, A.-C., **Martiny, A.C.,** Hoffmn-Bang, J., Ahring, B.K., and M. Kilstrup. 2003. Identification of bacteria from archeological wood. *In prep.*

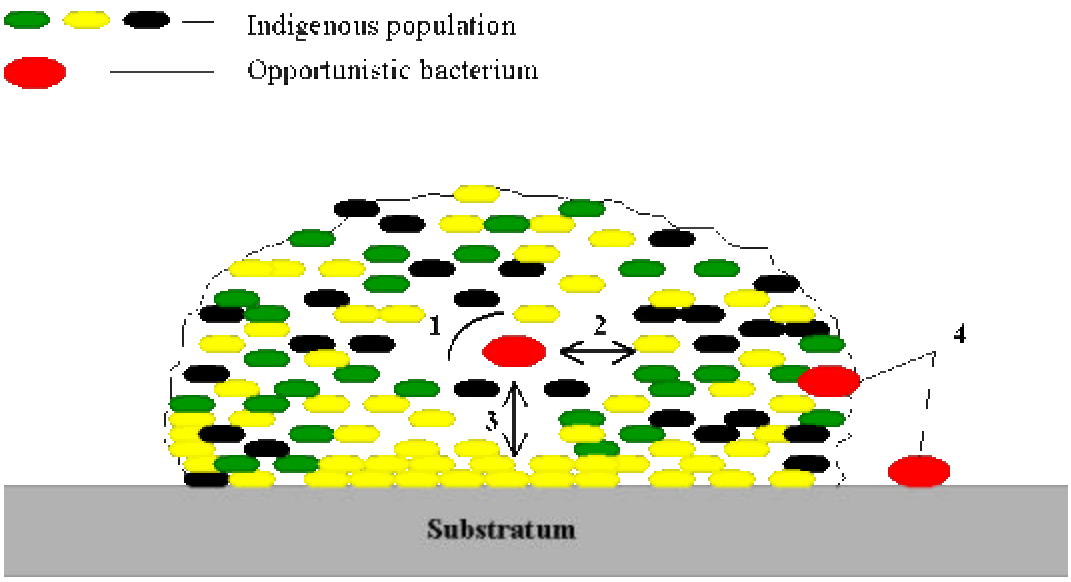
Bacteria related problems in drinking water

The issue of the microbial quality of drinking water has been a topic of great interest and concern since the mid-18th century. At this time, a connection between waterborne disease-causing bacteria and massive loss of human lives was made. This led to an increased attention of the importance of resource protection and careful treatment of raw water sources. Improved management resulted in a decrease in the number of pathogenic outbreaks reported until the 1960s, when the reported incidences raised again. The observation is explained as an effect of an improved reporting combined with increased pressure on water sources through urbanization, agriculture, and extended use of remote areas (110).

An intense monitoring of the quality of potable water has led to recognition of a number of problems related to the passage of water from the treatment plant to the consumer. The first problem to be documented was the occurrence of indicator bacteria, e.g. coliforms, in otherwise clean water that caused false alarms of contamination. The presence of indicator bacteria can be associated with bacterial (re)growth in the water downstream from the treatment plant, due to a lack of an effective disinfection residual, overabundance of nutrients supporting bacterial multiplication, or a combination of the two (75, 82). The monitoring of indicator organisms of external contamination is hampered, if coliform densities increase in the absence of infiltration. Secondly, regrowth of potential pathogens like *Aeromonas*, *E.coli*, *Legionella*, and *Pseudomonas* is a latent public health risk.

Another problem observed in water distribution systems is the presence of a microbial biofilm regardless of a (high) disinfection residual (125). The biofilm may act as a protective barrier against disinfection, allowing pathogens to survive, potentially multiply, and ultimately detach into the bulk phase. In addition, indigenous bacteria may produce a microenvironment more favorable for proliferation of opportunistic bacteria by providing nutrients needed for growth (103, 156). The source of nutrient can be excretions or leakages from bacteria or protozoa, remnants of lysed cells, and degradation products from extracellular decomposition of recalcitrant compounds.

Living in a biofilm may create alternative ecological opportunities for bacterial proliferation and survival. This disparity can result in a differentiation of the community composition between living in a biofilm and the bulk water (2, 67, 118) (paper 1, 2 & 3). In other words, bacteria in drinking water distribution systems proliferate in two different microhabitats. Both communities have an impact on the hygienic quality of the potable water; the bulk water because this is ultimately what the consumers use and the biofilm because of a possible role as a safe-haven for unwanted bacteria that later detach into the water phase of the distribution network. Therefore, to characterize the microbial community in drinking water distribution systems, it is necessary to include the populations from both sub-environments.



1. Protection from disinfection and shearing **2.** Cross-feeding or starvation **3.** Establishment of gradients e.g. anaerobic zones **4.** Differential attachment on abiotic and biotic surfaces

Figure 1. Potential factors linking the indigenous bacterial flora and the survival of opportunistic bacteria

Figure 1 summarizes potential factors affecting the survival and proliferation of pathogens in drinking water distribution systems. Although the nature of the pathogenic strain itself has a strong effect, the figure clearly illustrates many levels of interaction between the surrounding environment and a single cell, and thereby influencing the fate of an organism. Moreover, the local environment is affected by the presence of a microbial population, which then has a self-induced impact on the composition of the community (paper 4). Thus, to characterize mechanisms of survival of unwanted bacteria in drinking water distribution systems, one needs to understand the complex interaction between the local environment, indigenous microbial populations and pathogenic bacteria.

Measuring bacterial diversity in "natural" communities

The assessment of diversity in microbial communities is influenced by the approach applied for monitoring. No single technique will provide a complete determination of the diversity. Therefore, it is necessary to be aware of the bias and limitation of each individual approach before one can apply the results.

Culturing vs. non-culturing

No axenic bacterial cultures are naturally occurring in Nature. This fact is important to consider, when discussing how to analyze microbial communities. One can undertake two approaches. In the first approach, each member of the community is extracted and analyzed and the composition/behavior of the total population is consider to be the sum of all individual members. The second approach analyzes the community as an intact entity; no community trait is the result of individuals alone, but arises from interactions among different members of the microbial "society" (100). (There are pros and cons with both approaches, experimentally and conceptually that will become clear in the following discussion.)

Traditionally, researches have undertaken the first approach by isolating individual strains to pure culture and transferring them to the lab for further analysis. In particular, most of our knowledge arises from the systematic exploration of a few model strains, e.g. *E. coli* and *B. subtilis*. More than a decade ago, several researches discovered previously undetected high bacterial diversity in the environment (46, 161). Torsvik et al. measured 10^{10} cells and more than 4000 bacterial species (or at least bacteria with different chromosome) per gram of soil. These findings complicated the culturing approach.

The fraction of bacteria that is culturable in the environment usually ranges from 0.01% up to 10% and can be dependent on the physiological state of the cell (3, 109). In a study examining a drinking water system, the ratio of CFU pr cell (measured with Acridine Orange) was app. 10% in the initial colonization phase whereas in a mature biofilm, it dropped to 0.004% (11). Other factors e.g. oxidative and temperature stress, can also affect cultivability. As a result, this low and variable fraction makes cultivation unsuitable for enumeration of the total population in a sample.

The other issue with a culturing approach is, whether the determination of the composition of the community will be biased towards easily growing populations. Many authors have discovered that species found with plating and isolation on rich media are distinctively different from the bacteria detected with a genetic approach (54) (paper 3). In a study of a drinking water distribution system, Manz and co-workers found that a strain rarely found during cultivation dominated the *in situ* composition of the biofilm (63). But plating and isolation can still be useful when comparing the relative distribution of diversity among separate populations. In a comparison of four arid soil plots, the authors found that their "data illustrate, while 16S rDNA cloning and cultivation generally describe similar relationships between soil microbial communities, significant discrepancies canoccur". This was mainly assigned to "sampling different segments of microbial communities" (37). This correlation was also observed between the culturable and total population in the biofilm and bulk water in a Danish drinking water distribution system (paper 2+3). In comparison to many molecular techniques, cultivation underestimate the total number

of bacteria and mask for abundant species, but in some cases a similar pattern of distribution of diversity was observed.

Physiological traits

One of the advantages by culturing bacteria is the easiness, whereby one can examine various physiological attributes, e.g. as listed in Bergey's manual (9). Interesting traits could be carbon source utilization, growth rates, attachment rates, number of rRNA copies as a measure for adaptation to low nutrient environments (66), disinfectant resistance etc. These are all traits, which are difficult to assess independently of cultivation.

Some of these attributes can be assessed independently of cultivation. The carbon utilization pattern of bacteria can be measured with radioactively labeled substrates, followed by microautoradiography (MAR). This technique has successfully been applied to determine physiological traits in activated sludge communities operated under different conditions. These traits are then linked to diversity information with fluorescent probes (83). There are two major limitations of this approach in drinking water research. One is the difficulty of obtaining labeled substrates of ecological relevance, e.g. humic acids. The other is the restriction of only looking at monolayers of cells (and thereby not microbial aggregates like biofilms, unless homogenized (57)).

Another way of assessing physiological data without cultivation is by predicting traits from genomic data. A simple way is comparing 16S rRNA sequences with its nearest described (cultured!) neighbor. In some bacterial divisions this can reveal significant data regarding lifestyle (e.g. *Cyanobacteria*), whereas in other divisions it can be difficult to retrieve any information from this comparison (e.g. *Proteobacteria* or *Acidobacterium*). Among *Proteobacteria*, many closest neighbors have distinctively different properties, e.g. photo- vs. chemotrophy or auto- vs. heterotrophy, whereas only few described strain are affiliated to *Acidobacterium*.

A more elaborate approach is sequencing of larger gene-fragments extracted directly from the environment using bacterial artificial chromosome (BAC) libraries. Consequently, one can predict various functions by *in silico* analysis of the sequence and if the fragment contains a 16S rRNA gene or another known marker, assign it to a bacterial specie (143). This approach has successfully been used in finding an abundant un-recognized group of phototrophic bacteria in the Ocean (8), genes for new anti-microbial compounds (128), and estimating the diversity of aquatic viruses (12). Ultimately, the goal is to assess the complete genetic information in the environment, the so-called meta-genome (129). Proceeding on, people have cloned and expressed interesting genes found with this technique, and thereby extensively analyzed specific traits without requiring the given strain in culture (8, 128).

Measuring diversity using 16S rRNA

PCR based techniques

The most common way of accessing the diversity in a given environment is PCR amplification of 16S rRNA genes. Using PCR amplification to access the 16S rRNA gene pool can introduce potential artifacts and biases. There are several types of problems reported - the formation of heteroduplexes, chimeric sequences, differential amplification, and simple mistranslations (see (170) for a good review). These artificial sequences can cause an erroneous description of the diversity, since some sequences may not represent genuine species. Nevertheless, this technique has

been successfully applied in many studies to assess or compare diversity of various environments. It appears that a crucial step in using this approach is to carefully standardize every step and thereby reduce potential errors (112, 159).

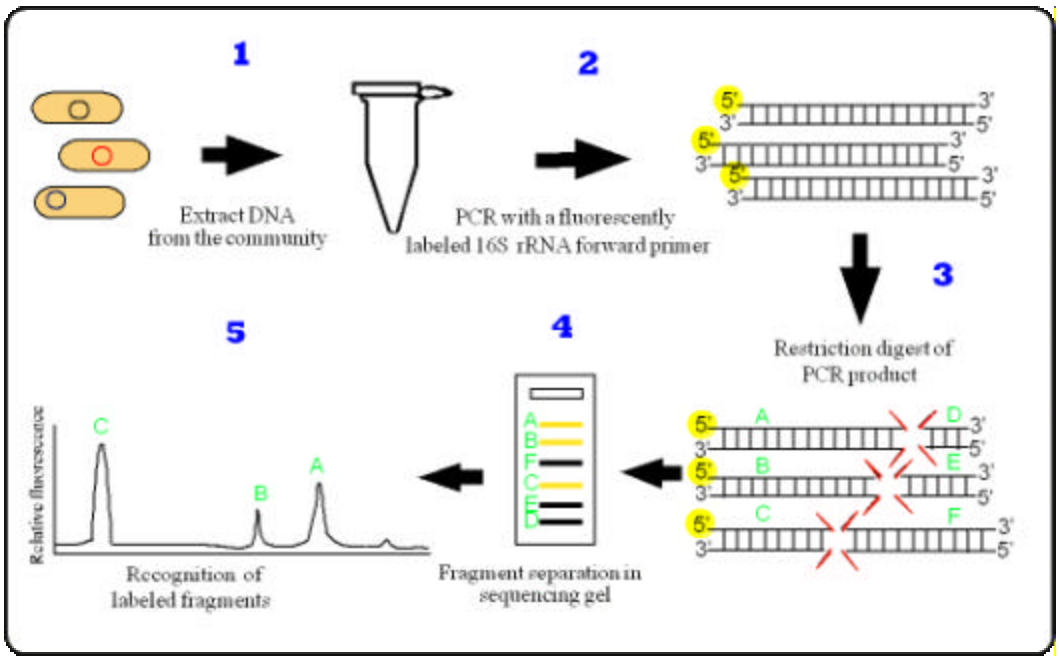


Figure 2. Principle of T-RFLP

One can divide PCR-based techniques for measuring the microbial diversity in a sample into two categories: those that provide a profile of the total community and those that measure the identity of each individual and then assemble the total community (a discrete dataset). Terminal restriction fragment length polymorphism (T-RFLP) analysis falls into the first category. T-RFLP measures the size polymorphism of terminal DNA fragments arising from a restriction of PCR amplified, fluorescently labeled 16S rRNA (or other genes) amplicons. Comparative genomics gives the insight to design the primer and choose the restriction enzyme. Automated systems like ABI can perform a precise fragment separation using capillary electrophoresis. The fragment length estimation becomes very accurate by adding differently labeled size markers together with the sample securing a high resolution of the technique (93).

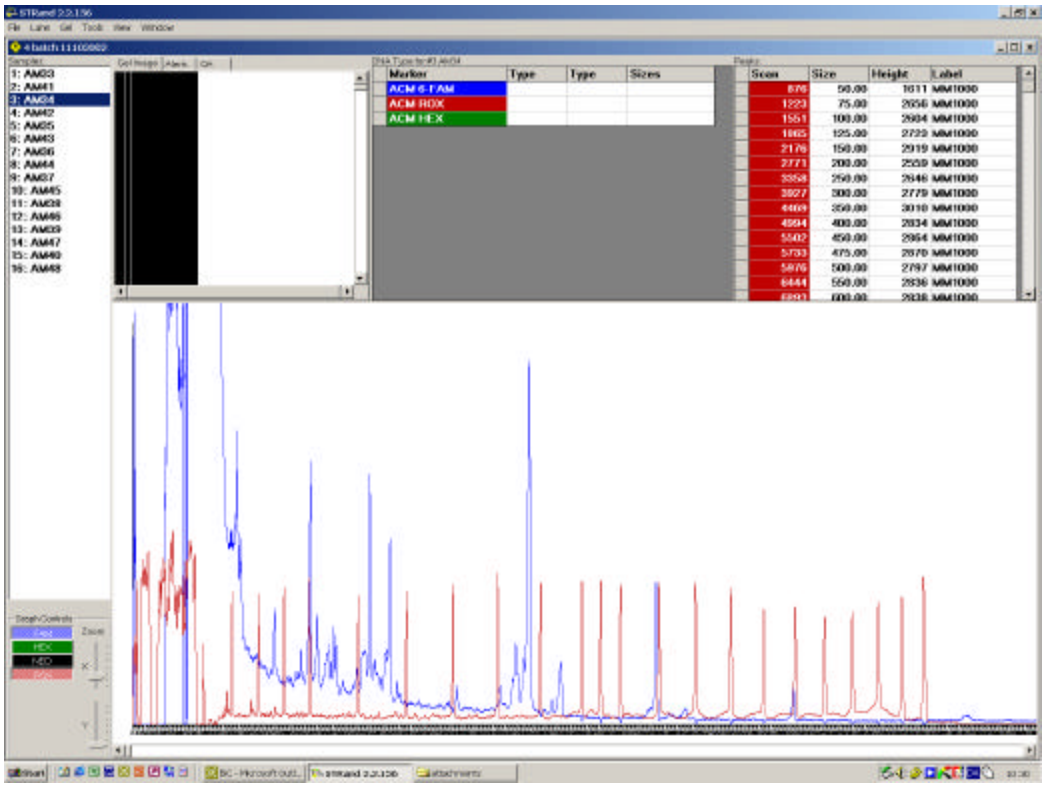


Figure 3. Screen shot from STRand showing a T-RFLP profile from a biofilm in a drinking water distribution system (blue) and a molecular weight standard (red). From paper 4.

Finally, a comparison between the observed fragment sizes in the sample and an *in silico* digestion of a relevant database can allow predictions of the identity of present bacteria (94). A profile of 16S rRNA fragments in a diverse environment will generate many peaks, which then has to be compared to the RDP database of more than 20.000 entries. Therefore, this prediction of identity based on the fragment size is only feasible, if the target population/gene has a relative small diversity, i.e. as seen with ammonium oxidizers (124).

With another community profile technique, denaturing gradient gel electrophoresis (DGGE), DNA fragments of identical lengths but with different base-pair sequences can be separated. Separation in DGGE is based on the decreased electrophoretic mobility of partially melted DNA molecules in polyacrylamide gels containing a linear gradient of urea and formamide. The melting behavior of amplicons are dependent on the strength of the attraction of the two DNA strands and thereby G-C vs. A-T content. Sequence variation will give rise to different melting behaviors and a polymorphism on the gel. Attaching a 40 base-pair GC-clamp on the amplicons can increase the resolution of the technique, so that the fragments only become partially melted, and migration is halted (137). Different approaches for post-staining of the gel can be applied including ethidium bromide, SYBR green/gold, and silver staining. Finally, individual bands can be excised and sequenced for further identification (104).

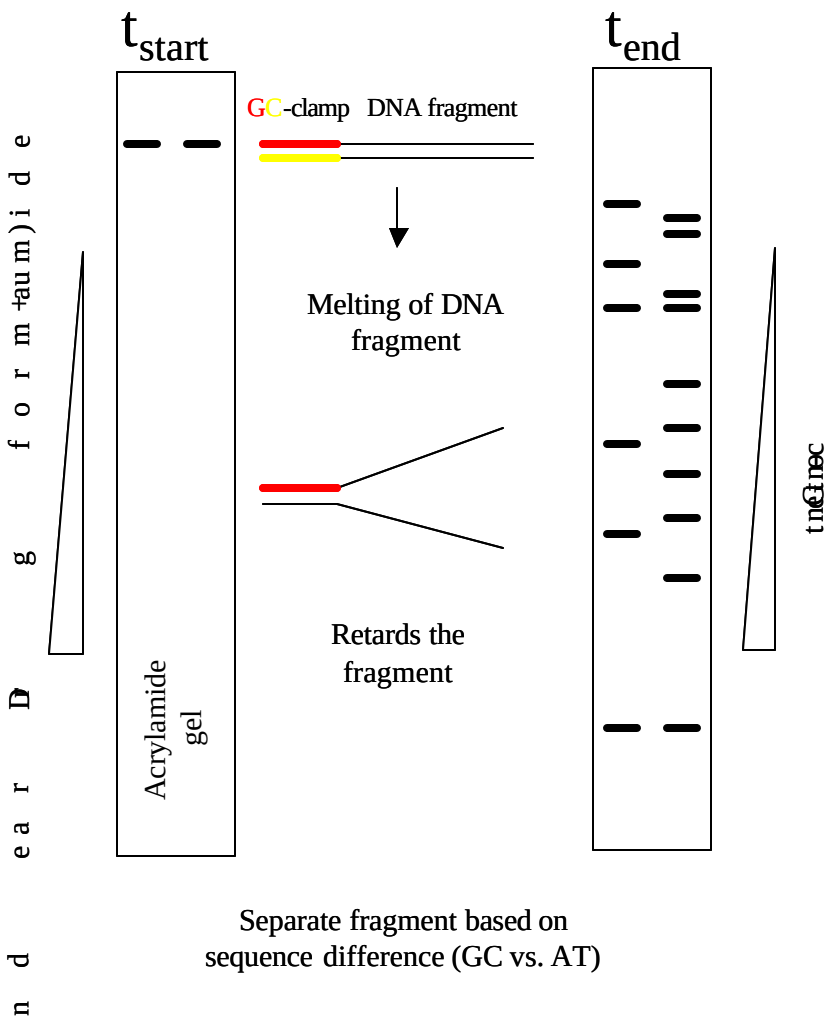


Figure 4. Principle of DGGE

Single stranded conformational analysis (SSCP) gives arise to a similar dataset as T-RFLP and DGGE. In SSCP, denatured PCR amplicons are electrophoresed through a non-denaturing polyacrylamide gel. The separated single strands adopt primary conformations that are dependent on their base sequence and determine the migration-rate in the gel. A single gene will result in two bands, one for each strand. A variation of the technique includes a step whereby one of the strands is digested using an endonuclease. This is done by phosphorylating one of the primers, which then become a recognition site for the endonuclease. This variation ensures each band in a profile arise from individual entries and thereby makes the interpretation of a profile from a mixed community feasible (136).

In contrast to community profiling, cloning of PCR amplified fragment allows one to assemble information about each community member one by one. By applying commercially available kits like TA cloning kit (Invitrogen, Carlsbad, CA) or pGEM-T (Promega, Madison, WI), individual PCR fragments is easily cloned and transformed, resulting in individual colonies. In this way, the analysis of the community is organized in a discrete dataset, which influence the downstream analysis. The individual clones can be analyzed in multiple ways. The easiest approach is by restriction analysis (amplified rDNA restriction analysis (ARDRA)),

using up to three different enzymes (102) (paper 1 & 2). A more elaborate (and expensive) way is by sequencing each clone (paper 3). The advantage by the second approach is the number of discriminative characters generated. ARDRA usually results in 3 – 10 bands (characters) per restriction enzyme, whereas sequencing results in 300 – 700 nucleotides (characters). The discriminative ability of sequencing is therefore 10 – 100 fold higher than ARDRA. Data generated from sequencing will also enable a tentative identification of the clones through a phylogenetic analysis.

In situ analysis

The basic principle of *in situ* analysis is to stain the bacteria followed by visualization of single cells in the microscope. Most bacteria cells have few discriminative morphological features, and as a consequence look identical in a microscope. There are two groups of stains, general ones that target a certain cell component e.g. DNA and RNA, and more specific stains that target a certain subpopulation. Many reagents for DNA staining are available (e.g. see www.probes.com). DAPI and acridine orange are commonly used for total counts. Acridine orange unspecifically targets a wide range of chemical compounds incl. RNA and proteins, resulting in a high level of non-target staining. In addition, DAPI cannot be visualized with a standard scanning confocal laser microscope (SCLM) set-up due to the low excitation wavelength. To overcome some of these problems, several SYTO stains can be applied. They have narrow excitation/emission spectra and fit the available lasers for optical sectioning with SCLM.

Fluorescent *in situ* hybridization (FISH) is based on the principle that two complimentary nucleic acids sequences hybridize and form a duplex. By designing the sequence of the probe, one can control, which cells are visualized. The most widely used target is 16S rRNA (101); rRNAs are present in 10^2 – 10^5 copy numbers, and therefore provide a natural amplification of the signal. *In situ* hybridization can be performed such that a sample is simultaneously examined with several probes marked with different labels. Following this approach one can start out using probes designed for the highest taxonomic level, the primary domains *Archaea*, *Bacteria*, and *Eukarya*, followed by probes specific for lower taxa (genus-, species-, and subspecies-specific). Another approach is to design several probes to target the same organism to control the specificity and to screen for closely related species (3). The result of FISH is a quantification of the present microbial community or a selected subpopulation as well as the spatial distribution of the microorganisms.

rRNA hybridization also provides a possibility of estimating the metabolic activity. Ribosome number and therefore the amount of rRNA reflects the growth rate, because the cell carefully adjusts and controls the content and synthesis of macromolecules to match the present environmental condition (134). The fluorescent signal intensity of the hybridized cell is proportional to the number of rRNAs molecules in the cell and will reflect growth rate at higher activity levels, whereas at low rates, a non-linear correlation between rRNAs and growth is seen (33, 34). Unfortunately, this is also a limitation of the method. At low cell growth rates or for cells with intrinsically low ribosome numbers (66), the signal intensity of the hybridized bacteria will be low. It may be difficult or even impossible to discriminate between background and a positive cell, and the technique may introduce a bias in the assessment of the community by selectively visualize active cells.

Several approaches have been developed to overcome this limitation including polylabeled ribonucleotides (32, 88, 116, 187) and signal amplification (117). These innovations have improved the sensitivity of FISH. However, the new approaches

have also introduced new limitations. It may be difficult to control stringency and specificity in a diverse community with polynucleotides. Currently, this approach has only been successfully used for separating deeply divergent archeal populations (32). Signal amplification relies on a sufficient cell-wall penetration, which can be difficult to achieve in mixed population, but the technique appears promising for planktonic communities.

The "Full Circle" analysis

The use of a variety of molecular techniques for describing the microbial diversity, abundance, and potentially physiological traits in the absence of any cultivation dependent step, has been adopted the "full circle" analysis (Figure 5).

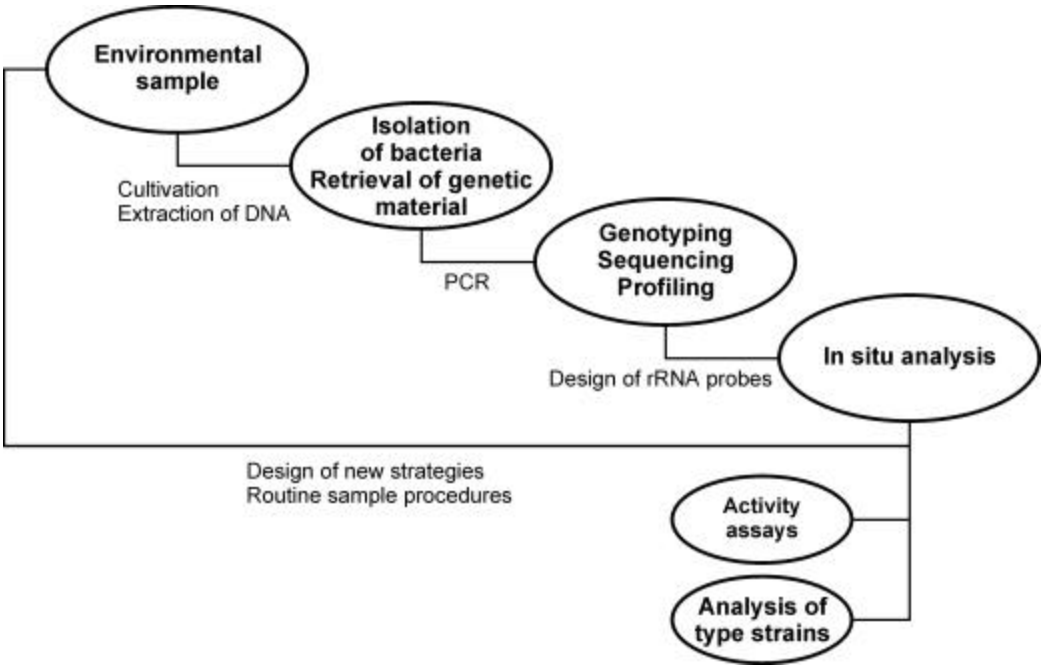


Figure 5. Diagram showing a complete investigation of an unknown microbial community. From Amann et al., 1995 (3).

The possible bias existing in profiling of PCR amplified fragments or isolation of strains for determining the microbial diversity, calls for an independent estimation of the distribution patterns. This has been made possible with fluorescence *in situ* hybridization, where the abundance of phylogenetic groups or specific strains can be estimated at a single cell level. A complete investigation of microbial diversity in an uncharacterized sample with a purely molecular approach should therefore start with extraction of total community DNA from the sample. The population can be qualitatively profiled using DGGE/T-RFLP and/or cloning and sequencing for a detailed phylogenetic study. Subsequently, the quantification of abundant taxa is achieved using fluorescent *in situ* hybridization. Finally, substrate utilization can be examined with microautoradiography or direct viable counts (63, 83).

Pros and cons with different techniques

Several approaches for analyzing the microbial community in drinking water have been tested during my three-year project. DGGE, T-RFLP and SSCP have been evaluated for an initial profiling of the population. T-RFLP proved to be a superior

peaks is usually detected, and it is difficult to mask any from the analysis, without seriously affecting the outcome. Regardless of approach undertaken, it will have an impact on the result.

Therefore, it is recommendable to employ cloning and sequencing to support a comparative analysis. This will provide absolute information of the microbial community (identity of species), whereas T-RFLP only gives a relative measure of the difference.

Cloning and sequencing is also a great tool for exploration of the diversity in a new environment. Additionally, the approach allows for an estimation of richness and abundance in a discrete manner, which is suitable for an ecological analysis of community distribution as discussed in the following chapter. Due to the large diversity in many environments, it is complicated to use this technique for comparative analysis unless a high number of clones are sequenced. Currently, this is both expensive and very elaborate to perform (imagine a sequence analysis of 10.000 clones!). So at present, T-RFLP would be recommended for comparative analysis and sequencing for a detailed analysis of dominant populations.

The second step in the analysis of a microbial community is the quantification of selected populations using fluorescent *in situ* hybridization. This technique has

worked well in particular in mesotrophic aquatic environments and activated sludge systems. In my own work, it was complicated by the combination of a low ribosome number and small cell size of the majority of the bacteria (paper 4). Therefore, to obtain structural information, DNA staining was employed, since it was not possible to visualize the different bacterial species *in situ* in the drinking water distribution system.

In general, drinking water in Denmark is characterized by a very low substrate level, which put some constraints on the molecular tools used for analyzing the bacterial population. As a consequence, FISH were not readily applicable for the analysis of this system, whereas T-RFLP and sequence analysis provided valuable information of the microbial community in a drinking water distribution system.

Comparing communities in space and time

The diversity in different habitats is a central theme in ecology. A common question is "how does the distribution of individuals affect ecosystem functioning?" and secondly "which processes control the distribution of individuals?". The previously mentioned techniques for identifying bacteria in environmental samples can quickly generate an overwhelming amount of data. In order to understand mechanisms governing the composition of communities, analytical tools are needed to deduct and recognize patterns affecting bacterial populations.

Diversity measurements

Diversity measurements consist of two components – the variety and abundance of species (with species meaning an operational taxonomic unit (OTU)). This information can be retrieved either by recording the identity and abundance separately (e.g. culturing/cloning followed by sequencing or ARDRA), or a measure that combines the two components (e.g. T-RFLP and DGGE). Many statistical indices and models have been proposed as a way of characterizing the community structure by a single number or distribution in order to facilitate a standardized way of comparing communities and adjust for uneven sample sizes. Species diversity measures can be divided into three main categorises; (i) species richness, (ii) species abundance, and (iii) species proportional abundance (91).

Index	Discriminant ability	Sensitivity to sample size	Richness or evenness	Widely used
a (log series)	Good	Low	Richness	Yes
l (log normal)	Good	Moderate	Richness	No
Q statistics	Good	Low	Richness	No
S (species richness)	Good	High	Richness	Yes
Shannon	Moderate	Moderate	Richness	Yes
Chao1*	Moderate	Low	Richness	No
Simpson	Moderate	Low	Evenness	Yes
Berger-Parker index	Poor	Low	Evenness	No
Shannon evenness	Poor	Moderate	Evenness	No

Table 1. Comparison of indices, from Magurran, 1988 (91), except * which is from Hughes et al., 2001 (55).

Table 1 lists different indices that are used to specify properties of the community e.g. richness or evenness. Zhou and co-workers applied Simpson's and log series (α) to characterize the influence of heavy-metal contamination and substrate level in soil on microbial population structure (186). They were able to quantify the community structure and illustrate patterns in the distribution of bacteria and demonstrate the value of using diversity indices in a characterization of communities. Additionally, a clear correlation between the two indices and therefore dominance and richness was observed.

Comparing communities

The diversity on a larger scale can be divided into categorizes describing the differences contained in the sample, between samples, and between communities (habitats), analogue to an analysis of variance.

Diversity	Description of diversity	Tool for quantifcation
α	Within-habitat	Diversity indices
β	Between-habitat or differentiation diversity (e.g. along a transect)	Cluster and principal component analysis or β diversity indices
γ	Overall diversity of a group of habitats with α-diversity	
δ	Between a range of regions (with γ-diversity)	
ε	Overall diversity of a group of habitats with γ-diversity	

Table 2. List of inventory diversity. From Magurran, 1988 (91).

Table 2 lists conceptual differentiation of inventory diversity by dividing it into α, β, γ, δ, and ε diversity, where α and β are the most commonly applied. The indices presented until now all describe the (α-) diversity of a sample (or homogeneous community). Often one is interested in comparing the change in the community composition along a gradient (e.g. spatial or temporal (paper 1, 2, 3, & 4)) or with chemical composition, all β-diversity. Again, new types of indices can be applied, but more commonly used is either cluster analysis based on similarity coefficients or ordination techniques.

Cluster analysis consists of two parts, a cluster criteria and a similarity coefficient. The clustering criteria can either be a simple pair-wise coupling like UPGMA or Ward's method (166) or more advances approaches taking the total dendrogram morphology into account as seen in phylogenetic analysis, e.g. neighbor-joining, maximum parsimony, or likelihood.

Similarity coefficients can either measure the presence/absence of species (qualitative comparison) or include abundance (quantitative comparison) in the samples. It is questionable, if a quantitative evaluation provides additional information in comparing microbial communities (10, 98). Several studies have shown how the cumulative peak height or band intensity in T-RFLP or DGGE can vary quite significantly (38) (paper 4) and inflect the quantification of individuals in the sample. Also, when applying cloning and sequencing, potential PCR biases can skew the ratio between the different species (154). These factors complicate the inclusion of abundance data in a cluster analysis.

Ordination techniques can be used to investigate the overall similarity of samples/sites and distinguish major groupings. The method does not provide a direct measure of β-diversity (e.g. similarity coefficient) but can be used to assess trends in diversity (identify principal components).

Characterizing both techniques are the absence of any statistical comparison to test for significant trends. One approach is extracting data from the original analysis (e.g. peak numbers, abundance of certain species etc.) and performing student's t-test on the average of each clade or group of selected characteristics. Another method compares a similarity matrix of the site data and resulting population profile.

Consequently, the correlation of matrices can be statistically tested and thereby provide a significance level for congruence in site data and resulting community profiles, as demonstrated in paper 4.

Diversity of bacteria and the species concept

The distribution of diversity is governed by the definition of a distinct individual in a given sample, an operational taxonomic unit (OTU). The most common unit for an OTU in microbial ecology is the ribotype, e.g. 97% 16S rRNA sequence similarity. So how well does a ribotype represent variability of diversity in a community? This question is linked to the understanding of taxonomy and concept of species in bacteriology.

Bacterial taxonomy is limited by the lack of sexual reproduction, a principle by which eukaryotic organisms are classified. Obviously, this concept does not apply to asexual organisms, and "the golden standard" in defining new species among prokaryotes is a genome similarity score of 70 %. Below this threshold, two individuals will be classified as two discrete species.

The application of primary sequence data to derive evolutionary descent of organisms and draw genealogical trees has had an enormous influence on prokaryotic systematics. 16S rRNA is the most popular marker for evolutionary descent (89), despite intense discussion on the impact of lateral gene transfer the last years (36, 139). Ribosomal genes have the advantage of including all living organisms as well as evolving slow enough to include ancient events (180, 181). Phylogenetic reconstruction provides an objective classification system, especially for taxa above species (174). It is important to note that in the absence of useful microbial fossil records, no time-scale of ancestry can be attached.

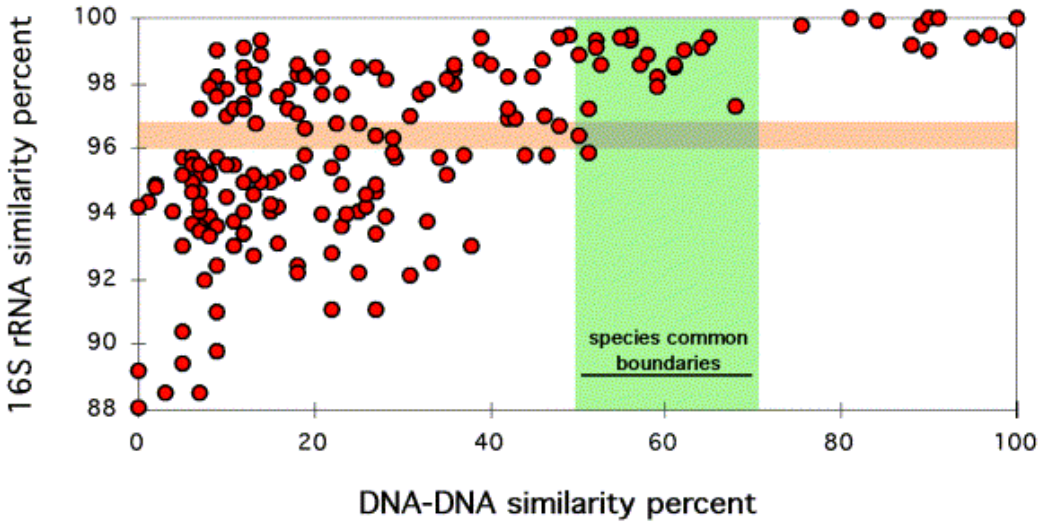


Figure 6. Comparison of DNA–DNA and 16S rRNA similarities, from Roselló-Mora and Amann, 2001 (130).

Stackebrandt and Göebel compared 16S rRNA and genome similarity to test if rRNA sequence similarity could be used to assign new species. They observed that 16S rRNA scores below 97% resulted in genome similarities of less than 70% and thereby different species (Figure 6) (141). Importantly, this does not apply the opposite way (a common misconception), and the authors concluded that 16S rRNA sequencing could only be applied to part species and not to cluster. Later, Rosselló - Mora and Amann confirmed this result using a larger dataset, and they recommended a polyphasic approach in which phylogenetic (16S rRNA) and phenotypic data is combined (130). The rationale is, that no gene will represent the full history of the

genome and in particular functional traits will be impossible to predict from e.g. 16S rRNA data (36, 73). In order to provide additional information regarding the organism, various individual discriminative phenotypic properties must be included (130).

For many years, it was impossible to determine physiological characteristics for a hierarchical discriminating and classification of environmental isolates, e.g. as illustrated with the growing number of organisms defined as *Pseudomonas* (182). Regardless of the heavy criticism of using 16S rRNA for estimating a phylogenetic relationship among bacteria, the conceptual idea of using this gene as the backbone for bacterial taxonomy has been very beneficial for identification bacteria in the environment.

Moreover, 16S rRNA sequence data does not provide the full picture of diversity, but the information included in 16S rRNA enables a discriminating of two organisms by sequence dissimilarity as well as provide an estimate of evolutionary relatedness. Combined with the option of retrieving this information in a culturing independent manner as described in the previous chapter, makes this marker suitable for a wide variety of ecological studies of microbial communities.

Finally, the application of 16S rRNA for ecological studies led to the recognition of previously unknown diversity, and thereby expanding our understanding of microbial life on Earth tremendously.

Opportunistic species	Environment		Surface material		Disinfection[a]			Detection			Reference
	Biofilm	Bulk	Steel/iron	Polymer	Cl_2	NH_2Cl	No dis.	Cultured	In situ	Dominant	
Acinetobacter	x	x	x	x	x		x	x	x		(81, 110, 158) (paper 2 & 3)
Aeromonas	x	x		x	x		x	x			(20, 23, 69, 78, 81, 113, 157, 158) (paper 2)
Alcaligenes		X			x			x			(81, 107, 110)
Bacillus		X			x			x			(81, 107, 110)
Campylobacter		x			x		x	x	x		(48, 133, 157)
Citrobacter		X			x			x			(23, 81, 110)
Corynebacterium		X			x			x			(81, 110)
E. Coli		x			x			x			(23, 53, 76, 81, 155)
Enterobacter		X			x			x			(23, 76, 81, 110)
Helicobacter		x					x	x			(56, 157)
Klebsiella		X			x			x			(23, 81, 110)
Legionella	x	X		X	x		x	x	x		(39, 110, 151, 160, 172) (paper 2)
Micrococcus		X			x			x			(81, 107, 110)
Moraxella	x	X	x		x			x			(81, 110, 158) (paper 2)
Mycobacterium	x	x	x	x	x		x	x			(47, 74, 107, 157, 169, 172)
Nocardia	x	x	x	x	x			x			(107)
P. aeruginosa	x	x			x			x			(81, 110, 157)
Salmonella		X			x			x			(110)
Serratia		X			x			x			(81, 110)
Shewanella	x	x	x				x	x	(x)		(90) (paper 2)
Shigella		x			x			x			(177)
Staphylococcus		X			x			x			(81, 110)
Streptococcus	x			x	x			x			(172)
Yersinia		x			x			x			(72, 81, 157)

Table 3. List of opportunistic bacteria in drinking water. The list is only tentative since many bacteria has been renamed or a inadequately indentified.

[a]Disinfection type in system where bacterium is detected

[b]Detected with cultivation independent technique, e.g. PCR or hybridization

Genus	Environment		Surface material		Disinfection			Technique			Reference
	Biofilm	Bulk	Steel/iron	Polymer	Cl_2	NH_2Cl	No dis.	Cultured	In situ [b]	Dominant	
Achromobacter		X			x			x			(110)
Acidobacterium	x	x	x				x		x	x	(paper 3)
Acidovorax/ Methylomonas	x	x	x		x		x	x	x		(107) (paper 2 & 3)
Agrobacterium	x	x		x	x			x			(107)
Aquabacterium	x	x	x	x			x	x	x	x	(61, 63) (paper 1, 2, & 3)
Arthrobacter		X			x			x			(81, 110)
Bacteriodetes	x	x	x				x		x		(paper 3)
Bradyrhizobium		x					x	x			(65)
Caulobacter		x					x	x			(157) (paper 1 &2)
Chlorobium	x		x				x		x		(paper 3)
Comamonas (B3)	x	x		x			x		x		(61, 63)
Devosia	x	x	x				x	x	x		(paper 2 & 3)
Flavobacterium	x	X		x	x			x			(81, 110, 158)
Fusobacterium		x			x			x			(172)
Gallionella	x		x		x			x			(125)
Hydrogenophaga	x	x	x	x	x		x	x			(107) (paper 2)
Lactobacillus	x	X		x	x			x			(110, 172)
Leptothrix	x	X			x			x			(110, 125)
Nitrobacter	x	x		x		x		x	x		(123, 124)
Nitrosomonas	x	x	x	x		x	x		x		(123, 124) (paper 3)
Nitrosospira	x		x				x		x		(paper 3)
Nitrospira	x	x	x	x		x	x		x	x	(123, 124) (paper 3)
Ochrobactrum	x	x		x			x	x			(158)
OP12	x		x				x		x		(paper 3)
Pasteurella	x	x		x			x	x			(158)
Pedomicrobium		x						x			(157)
Pirellula	x		x				x		x		(paper 3)

Table 4a. Tentative list of bacterial species in drinking water

Genus	Environment		Surface material		Disinfection			Technique			Reference
	Biofilm	Bulk	Steel/iron	Polymer	Cl_2	NH_2Cl	No dis.	Cultured	In situ	Dominant	
Planctomyces	x	X	x		x		x	x	x	x	(110) (paper 3)
Pseudomonas	x	X	x	x	x		x	x	x		(81, 90, 107, 110, 158) (paper 2 & 3)
P. fluorescens		x			x			x			(172)
Rhodobacter	x	x	x				x		x		(paper 3)
Rhodocyclus (B2 B5)	x	x					x	x	x	x	(61, 63) (paper 1 & 2)
Rhodoferax		x					x	x			(paper 2)
Salinococcus	x		x				x		x		(paper 3)
Sarcina		X			x			x			(110)
Sphingomonas	x	x	x	x			x	x	x		(68) (paper 1, 2 & 3)
Stenotrophomonas	x	x	x	x	x		x	x			(65, 107)
Telluria		x					x		x		(paper 3)
Thiobacillus	x	x	x				x		x		(paper 3)
Variovorax (B7)	x						x	x			(63)
Verrucomicrobium	x		x				x		x		(paper 3)
Xanthobacter	x	x	x	x			x	x	x		(65, 107)
Xanthomonas	x	x	x				x	x			(65) (paper 1 & 2)
Zoogloea (B1)	x			x			x	x	x		(61, 63)

Table 4b. Tentative list of bacterial species in drinking water

Bacterial species in drinking water

Since the discovery by John Snow of a connection between outbreaks of cholera and handling of drinking water wells in London in 1854, numerous studies have examined the diversity of microbes present in distribution systems and their impact on public health. Most research has focused on defined groups of bacteria of hygienic interest (e.g. pathogens). Table 3 lists commonly encountered opportunistic species and where and how the bacteria have been detected.

Potential pathogens

Modern concepts of source water protection have virtually eradicated classical pathogens in developed countries, including *Salmonella typhi*, *Shigella* spp., and *Vibrio cholerae*. Nevertheless, a variety of potential pathogens are still encountered in water supply systems incl. *Aeromonas*, *E. coli*, *Legionella*, and *P. aeruginosa* (157).

The significance of *Aeromonas* spp. in drinking water in relation to gastrointestinal diseases is not clear (131). A correlation between *Aeromonas* possessing virulence factors and diarrheal cases was observed in water supplies (15, 69). Nevertheless, different aeromonads are regularly located and potentially proliferating in distribution systems, without being a source for infections (e.g. paper 2 and (81, 157)). Due to the ability of proliferation at very low concentrations, regrowth can also occur in drinking water (162).

E. coli occurrence has routinely been monitored in potable water as an indicator for fecal contamination, but even some serotypes of *E. coli* can present health problems (53, 155). In Walkerton, Canada, *E. coli* O157:H7 was responsible for one of the most serious outbreaks, in which bacteria originating from drinking water were involved. During this outbreak, 7 people died (53). *E. coli* strains have been shown to proliferate under conditions similar to drinking water (18, 156), but it is still unclear how enterohemorrhagic serotypes (e.g. O157:H7) proliferate in drinking water due to a large chromosomal difference to non-pathogenic relatives (115). The Walkerton incidence provides strong evidence of at least survival of these pathogenic strains.

Legionella pneumophila was discovered in 1976 as a consequence of an outbreak, in which a number of U.S. veterans died at a hotel in Philadelphia. Little is known about sporadic occurring legionellosis that accounts for the majority of infections, but correlation analysis indicates a link to residential water supplies (153). *Legionella* is naturally occurring in aquatic environments (43, 44), which then act as inoculum and cause a high number of domestic water systems to harbor the bacterium (131). Biofilms in dental air units is a particular problem, because *Legionella* can proliferate in the system and through aerolization cause infections during dental procedures. The bacterium is detected in the majority of the investigated dental systems and often in high density (4, 172).

An important trait of this organism is the capability of intracellular existence and multiplication. *Legionella* is capable of multiplying intracellularly in a wide range of protozoan hosts. This is the only described mean for growth of *L. pneumophila* in distribution systems. *Legionella* exhibits a physiological shift upon invasion and increases resistance to temperature, biocides etc., which can facilitate survival in the environment. In addition, following release from a protozoan host, *Legionella* displays an enhanced virulence to mammalian cells (1). In absence of an protozoan host, *Legionella* has not been shown to proliferate in drinking water (43), but can

persist for extended periods of time (59), in particular in the presence of a biofilm (103).

Currently, *P. aeruginosa* is among the most important opportunistic pathogens causing infections in immune-compromised or cystic fibrosis patients and patients with underlying diseases like wounds or pneumonia (27). It is the most frequent source of infection of burn patients, second leading cause of nosocomial pneumonia, the third most common cause of nosocomial urinary tract infections, and the fourth leading cause of surgical wound infections (131). However, healthy persons are rarely affected by this bacterium. *P aeruginosa* has been shown to contain a large number of genes involved in both regulation and catabolism enabling them to adapt and persist in many different environments (152). The most common mode of infection is to colonize a surface and form a biofilm followed by an over-production of alginate (51). The bacterium is regularly detected in drinking water and is able to proliferate at low substrates concentrations (164).

A general trend in the survival and potential multiplication of pathogenic bacteria is the ability to and/or need for interaction with the surrounding population (16, 103, 156). The indigenous population may act as a source for growth requirements or a protective environment against disinfection or predation. This points towards a complex relation between opportunistic and autochthonous bacteria, influencing the hygienic quality of drinking water.

Non-pathogenic strains

In order to understand and control the survival of pathogenic bacteria in drinking water, one therefore need to understand the distribution and physiology of the indigenous population, since this ultimately affects and to a great extent controls survival of unwanted organisms. The question is, if there is a pattern in which bacteria is detected in drinking water. Does certain groups of organisms that are continuously detected in drinking water, possess genetic information suitable for proliferation in this nutrient depleted environment? A variety of bacteria are routinely isolated from water supply systems (Table 4). This includes *Pseudomonas*, *Caulobacter*, *Sphingomonas*, and bacteria related to the family *Comamonadaceae*.

Pseudomonas is a common inhabitant of distribution systems. They readily form biofilms on many different surfaces as a strategy for survival in hostile environments and may act as opportunistic pathogens. *Pseudomonas* species are ubiquitous in water supply systems (110) and proliferate on a wide variety of substrates, and in particular oxidized compounds (164) (paper 2), and some species are used for measuring bacterial regrowth potential in drinking water (163).

Caulobacter and the closely related *Brevundimonas* have been shown to be ubiquitous in aquatic environments. A complete genome analysis of *Caulobacter cresentus* has shown that the genome contains multiple clusters of genes encoding for functions enabling survival in nutrient depleted environments (106). *Caulobacter crescentus* has more genes coding for nutrient uptake (active transport systems) in comparison to metabolism, indicating that substrate scavenging is important for proliferation of this organism. The detection of this species in drinking water is in good accordance with the theory that multi-substrate collection and usage is important for survival under oligotrophic conditions (17).

The genera of *Sphingomonadaceae* have capabilities to degrade a variety of refractory compounds (71) as well as optimizing their protein synthesis for growth under oligotrophic conditions (42). A dominant bacterium in marine environments, *Sphingomonas alaskensis* (167), has only one rDNA operon resulting in a very low

number of ribosomes per cell, ranging from 200 to 2000, resulting in a low maximum growth rate (42, 66). Strains from these genera have repeatedly been isolated in drinking water supply systems (68) (96)(paper 1 & 2), supporting their omnipresence in this oligotrophic environment.

Hydrogenophaga from the family *Comamonadaceae* was found to be the most frequently isolated strain in the bulk water in a Danish water supply system, but could also be detected in Berlin (paper 2). High sequence similarity scores to *H. pallerioni* were found for two of the genotypes included in this genus. In a recent study, bacteria closely associated with this species were found to dominate the microbial community in a sand-packed column perfused with groundwater (84). Similarly, the raw water source for the two examined distribution systems is filtered groundwater, suggesting that the presence of *Hydrogenophaga* could originate from the sand filter at the water work. In another study *Hydrogenophaga* was found in the raw water as well as on pipe surfaces of different materials (107). Other species affiliated to *Comamonadaceae* are phenotypically related to *Hydrogenophaga* by displaying a heterotrophic metabolism in nutrient depleted environments. Therefore, it is not feasible at present to predict the occurrence one species against another.

In situ abundance

It is clear from the table that most bacterial isolates found in water supply systems are affiliated to the phylum *Proteobacteria*. Does this reflect the true (*in situ*) composition of microbial communities in drinking water?

Several studies have applied group-specific hybridization probes in order to classify abundant microorganisms in drinking water. These studies clearly demonstrated a limitation of the traditional *in situ* hybridization protocol, since only a fraction (23 – 40%) of cells in the bulk water phase could be hybridized (62) (92) (paper 1), resulting in unreliable data for this subpopulation. More successful was the investigation of a young biofilm, where bacteria affiliated to various subclasses of *Proteobacteria* dominated the bacterial population. The results showed a high abundance of bacteria from the β-subclass during the initial colonization of the surface (62) (paper 1), even though the surface material and source water affected the distribution among the different subclasses of *Proteobacteria* (63, 135).

Werner Manz and co-workers proceeded by culturing and analyzing a number of strains on R2A medium belonging to the β-*Proteobacteria* in order to further identify the abundant organisms in drinking water. By verifying the individual abundance of the strains with *in situ* hybridization, they demonstrated; (i) a group of bacteria belonging to a novel genus, *Aquabacterium*, dominated the biofilm and (ii) that these bacteria grew on R2A (61, 63).

A clear limitation of the above-mentioned studies was the age of the biofilm. Most of the result arose from biofilms ageing 1-2 weeks, which potentially have an influence on the composition of the biofilm. In order to assess the influence of age as well as thorough mapping of the microbial population in bulk water and biofilm, a number of clones generated from an extraction of DNA and subsequent PCR of 16S rRNA were sequenced in this study (paper 3). The analysis showed an unrecognized diversity in drinking water including bacteria from the phyla *Acidobacterium*, *Planctomyces* and *Nitrospirae*. The bacteria affiliated to any non-proteobacterial phylum constituted 60 – 87% of the diversity depending on sample age/or sub-environment (bulk vs. biofilm). The result is therefore in contrast to previous reported studies on drinking water in which *Proteobacteria* dominates the biofilm.

Prediction of bacteria species in drinking water

Using the numerous studies of the microbial diversity of drinking water, can we now predict the microbial community composition based on the physio-chemical data of the drinking water distribution system? The truth is, that we have a limited knowledge (if any) on factors determining microbial diversity in the environment at present! There are two factors involved in this lack of understanding. One is the vast number of species co-existing in each little niche on Earth (e.g. as in a gram of soil (161)). Secondly, phyla like *Acidobacterium*, *Plantomycestes*, or *Verrucomicrobium* only contains a few described strains, even though sequence data from these groups are retrieved repeatedly in a wide range of environments and harbors as much genetic, and probably metabolic, diversity as *Proteobacteria* (7). These issues make it difficult to predict diversity in any environment.

Nevertheless, I believe that a number of species will continuously be observed in drinking water. *Pseudomonas*, *Sphingomonas*, *Aquabacterium*, and *Caulobacter* have all repeatedly been detected in drinking water (or related environments) and in some instances independently of cultivation (63, 167) (paper 3). Besides *Aquabacterium*, the mentioned organisms are all described as metabolic versatile and well adapted for growth in environments with a lower and changing amount of nutrients. It is doubtful that any of these strains will dominate an established community, but more likely be involved in responses to nutrient alterations or stress (e.g. chlorination).

Another group of organisms that is expected to be present in biofilms in drinking water is autotrophs. Systems characterized by a low biologically available carbon concentration may force the bacterial population to explore alternative energy sources. Recently, several studies have described the abundance of ammonia and nitrite oxidizers in water supply systems (86, 124) (paper 3). The formation of large iron tubercles may also create anaerobic zones supporting sulfate- or iron-reducers (5). Finally, bacteria related to *Acidobacterium*, *Planctomycetes*, and *Verrucomicrobium* are common in soil communities. Therefore, I think these groups of bacteria will constitute frequent and abundant populations in groundwater supplied distribution systems.

A number of recent studies have made considerable progress in the isolation of previously non-culturable organisms (25, 60, 185). The approach undertaken by Steve Giovannoni and co-workers of applying a high-throughput system to test for growth dependency of multiple parameters has enabled a thorough description of growth requirements for bacteria abundant in the oceans. Another approach involves a diffusion chamber in order to produce environmental growth conditions without any knowledge *a priori*.

It is likely that similar approaches will result in cultivation of abundant bacterial lineages and thereby provide additional information of the connection between phylogeny and function and enable us to make better predictions in the future. Bacteria expected to dominate a drinking water distribution system will include properties of an extremely small size ($D < 0.1$ µm) and an effective substrate uptake system in order to possess the highest fitness in this system (17), whereas a detailed phylogenetic relationship is unknown at present.

Organization of communities – how do bacteria interact inside a community?

As mentioned earlier, the bacteria living in a water distribution system proliferate in two niches, as planktonic or attached on surfaces as biofilms. Direct observations of a wide variety of natural ecosystems have established, that the vast majority of bacteria grow within a matrix-enclosed biofilm (for a review see (26)). This aggregation on surfaces creates self-imposed gradients leading to formation of ecological niches. Therefore, bacteria living in a biofilm experience a profoundly different environment than *most* planktonic bacteria.

Models for biofilm formation

The establishment of a biofilm is characterized by a step-wise process, where cells initially colonize the surface, followed by growth and formation of stacks of bacteria. Based on laboratory systems consisting of one or two species as well as mathematical simulations, several models accounting for the development of a biofilm have been proposed.

The first model is based on the idea, that each step in the formation of a mature biofilm and subsequent sloughing involves specific genetically controlled programs. The first step is attachment mediated by adhesion factors incl. flagellum, type IV pili, antigen 43 etc. This is followed by surface motility (e.g. twitching) and growth leading to microcolonies. Finally, differentiation from microcolonies into elaborate structures (e.g. mushrooms) is a consequence of different genetic pathways controlled by quorum sensing among others (29, 176). Other regulators result in disintegration of microcolonies and mobilization of planktonic bacteria (120, 150).

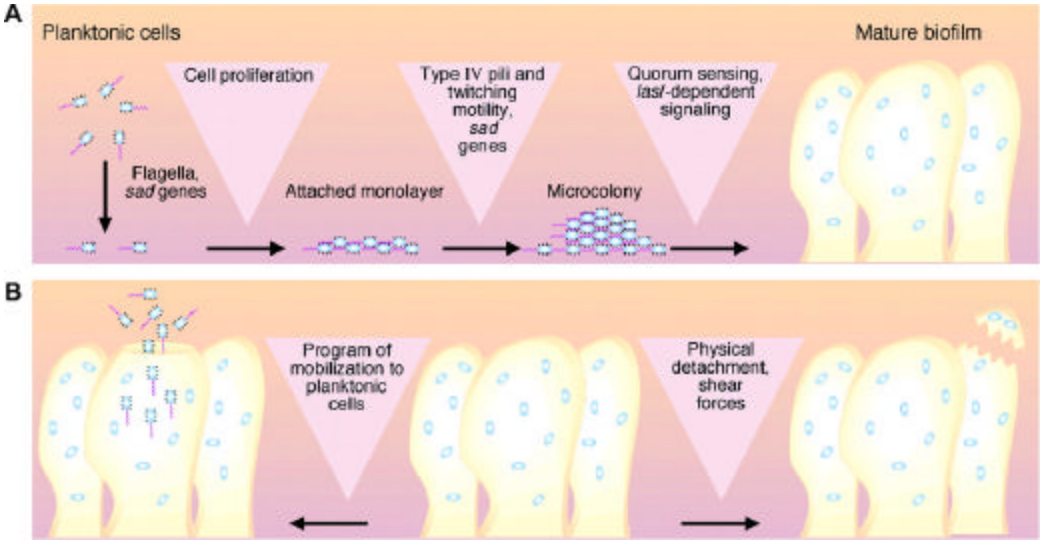


Figure 7. Model for a genetic pathway for biofilm formation. From Costerton et al., 1999 (27).

Another model proposes, that the structure of the biofilm is a predictable consequence of the physiochemical conditions in the surrounding environment (179). Wimpenny and Colasanti calculated with a cellular automaton model, that low substrate concentration resulted in isolated colonies scattered on the surface (towers) as seen in some drinking water systems (173) (paper 1 & 4). Intermediate concentration resulted in a more structured biofilm incl. "mushrooms" interspersed by

water channels, whereas a high concentration produced a thick mat. Van Loosdrecht and co-workers proceeded using a combination of a discrete and continuum model, in which "real" biological parameters were in use (119). The results show that mathematical simulations based on simple assumptions regarding growth, nutrients, mass-transport and detachment were able to predict a variety of biofilm morphologies as seen in Nature (178).

Factors influencing the biofilm structure

Assuming that microbial fouling of pipe surfaces in drinking water distribution systems are impossible to avoid, it is relevant to identify factors that influence the development and composition of the biofilm. Secondly, since we currently only have a limited grip on the species diversity in complex microbial communities as described earlier, any genetic traits involved in fouling of water supply systems can only be predicted from model communities.

Substratum

The influence of substratum composition on biofilm formation has been examined in a number of studies. The colonization is consequence of a transport to the surface due to diffusion, convection, or active movement. The next step is a reversible adhesion, which mainly is a physio-chemical process; followed by a biologically mediated irreversible attachment, incl. factors like flagellum, type IV pili etc. (165). A biological effect may be more pronounced at a latter stage of the biofilm formation when cells undergo a change from reversible to irreversible attachment and start to form microcolonies (52, 95).

Several studies looked at the influence of surface material on the composition of a natural microbial community in drinking water. Rogers et al. observed a limited colonization of *Legionella* on copper surface in comparison to various polymeric materials, whereas the total density of bacteria seemed unaffected (127). Manz and co-workers observed a similar trend (63, 135), even though a specific community response to soft-PVC was reported (61). Arvin and co-workers demonstrated how different phenolic additives migrated from PE tubing into the bulk water, and these organic compounds can act as substrate for bacteria (13). The potential leaking of compounds from substratum can attract or support certain bacterial species and influence the density and distribution of microorganisms in the biofilm.

An open question is whether surface material will have a long-term impact on the community composition in drinking water. No strong response has been reported yet. Secondly, most research were based on observations of up to 14-day-old biofilms and properties of the surface may change, when the bacteria is close to cover 100% of substratum as seen in mature biofilms (67) (paper 4). On the other hand, visual observations clearly demonstrate how iron pipes somehow attract iron precipitation creating an extremely rough surface in comparison to polymeric materials (personal observations) and this could have an impact on the composition of the biofilm.

Metabolic interaction

An advantage for bacteria in a biofilm is the high density of cells enabling metabolic cooperation in order to degrade recalcitrant compounds that are most likely to constitute a majority of the available carbon in a groundwater supplied distribution system. This metabolic commensalism has been described in several instances in which bacteria form a coordinated mixed community in order to optimize utilization

of available energy sources (21, 70, 105). Also in the syntrophic relationship in various anaerobic biofilm communities, bacteria organize along substrate gradients in order to create favorable thermodynamic conditions for growth (49, 111). In both examples, organization in biofilms creates opportunities for proliferation that are not present in a planktonic environment (26).

Disinfection resistance

One of the initial concerns of the establishment of a biofilm in a water supply system was the possibility of creating a safe-haven for opportunistic microorganism positioned inside the biofilm. Some early studies reported a reduced susceptibility to antimicrobial agents incl. antibiotics and disinfectants of bacteria attached to surfaces, enabling them to survive concentration significant higher than their planktonic counterpart (14, 77, 79, 121).

One hypothesized resistance mechanism is that the extracellular polymeric matrix of the biofilm retards the diffusion of disinfectants. It has been demonstrated that diffusion of small solutes in a biofilm is comparable to diffusion in pure water, and it is therefore difficult to argue directly for physical barrier (149). Nevertheless, a reduced concentration of biocides in the lower parts of the biofilm has been reported (30, 147). If the disinfectant is reactively neutralized in the biofilm like chlorine, incomplete penetration can occur. Bacteria and extracellular material constitutes a barrier exhausting the biocide at the upper part of the biofilm and thereby protect the deeply embedded bacteria.

Another hypothesis explain increased resistance as a result of a metabolic and physiological differentiation in the biofilm compared to a planktonic state. As an example, *P. aeruginosa* activates an array of genes upon attachment to a surface and during formation of a biofilm (132, 176). The idea is, that a number of genes affecting biocide resistance are up-regulated in the biofilm, enabling the cells to survive disinfection. These genes can include extracellular polymer production (28, 51), enzymes excretion involved degradation of biocides (6, 148), rapid spread of resistance genes (22, 87), and induction of a general stress response, e.g. due to localized zones of reduced metabolic activity (144, 184).

Stewart and co-workers compared the impact of two sigma factors on the resistance of *P. aeruginosa* to biocides in biofilm and bulk water. These were regulators of EPS production (*algT*) and starvation response (*rpoS*). The authors demonstrated a reduced susceptibility to monochloramine and hydrogenperoxide in the biofilm, whereas *algT* and *rpoS* mutants only transiently suppressed resistance to the biocides. In young thin biofilms an effect was detected, whereas no contribution was provided in older, thicker biofilms (24). This suggested that the primary mechanism for resistance was the biofilm acting as a reactive barrier to disinfection.

The mechanism of increased resistance in a biofilm towards antibiotics is similar to the resistance against biocides, including failure to penetration, an inherent resistant phenotype, and substrate gradients leading cells to enter a non-growing state (145). A debatable issue is whether this increased resistance is inherent to the biofilm or an effect of a changed physical environment. Recently, Lewis and co-workers showed that non-growing cells had an equivalent survival-rate to a wide variety of antibiotics. This suggested that regardless of microenvironment, the physiological state of the cell is an important factor for resistance (140).

At present it is unknown if the microbial population in drinking water will possess a differentiated resistance to disinfection and as a result alter the community after biocide treatment. Nevertheless, it appears that reduced susceptibility of bacteria

in response to disinfection using oxidants as chlorine or ozone is primarily due to the biofilm imposing a reactive barrier and thereby depleting the disinfectant (146), but an influence of a physiological response cannot be rejected.

A secondary effect of disinfection is the increased concentration of nutrient due to oxidation by chlorine or ozone, supporting microbial growth (41, 82). A hypothesis is that strains adapting fast to changes in nutrient concentration are abundant among pathogens, since this is a common part of their lifestyle. Therefore, disinfection may cause some unwanted negative hygienic effects, by creating an unstable community with too few cells compared to the available nutrients and selecting for fast growing cells that proliferate when the biocide is depleted.

Other factors affecting biofilm formation

Several other factors influence the diversity of bacteria in the biofilm. Protozoans are present in biofilms in nearly all water distribution networks including the ones analyzed in this study (45, 62, 96) (paper 1)(138). Microcolony formation may protect against grazing, since microcolonies "can be too big of a mouthful" for protozoans (97). In contrary to that observation, an increased grazing activity has been measured on substratum compared to the bulk water phase (19, 138). Moreover, protozoans were observed grazing on microcolonies in a biofilm formed in flow-cell using drinking water as inlet water (paper 1). The observations indicate at complex interaction between different trophic levels, that potential can affect the community composition in the distribution system (138).

The flow-rate have been demonstrated to have an impact on the architecture of the biofilm and influence potential signaling between the cells, e.g. quorum-sensing (114, 119, 122). The influence of flow-rate may partly be contributed to a higher convective transport of nutrients into the biofilm and as a consequence a higher effective substrate concentration observed by the cell (119). The level of energy source and electron acceptor does clearly affect the structure and diversity of the biofilm community (31, 40, 64). A temperature difference will also influence the microbial activity in the drinking water distribution system and as a consequence pertubate the community structure (82, 127).

The influence of activity on biofilm speciation

Besides from specific factors inducing a define effect on the biofilm, the simple fact of being fixed on a surface enables the community to develop in space and time. The species diversity of natural communities is often strongly related to the growth rate in the system (often termed productivity). Nonetheless, no general consensus concerning the form of the pattern has emerged. Some studies show an enhancement of richness as the productivity increase, other a negative effect and finally some describe it as unimodal.

Studies describing a positive relationship explain it as an effect of more dynamic systems (e.g. have more available energy) and thereby creating room for a higher richness. Mechanisms thought to reduce diversity include the idea of competitive exclusion, whereby the fittest organisms outcompete the rest. Another theory justify it as evolutionary immaturity. In the excess of nutrients, community members do not need to evolve in order survive, with the result of creating a system of relative low diversity. This mechanism may not apply to prokaryotes due to the fast exhaustion of any available energy in a system. A unimodal pattern is usually explained as a trade-off between different factors influencing diversity including growth rate and resistance to predation as well as the creation of niches (a

heterogeneous environment). Importantly, the spatial scale of the system exhibits a strong influence on productivity-diversity relationship. At low scale, the pattern is commonly unimodal, whereas the diversity changes monotonically across larger areas (171).

Rainey and co-workers studied growth of *P. fluorescens* in laboratory microcosms at different nutrient concentrations as a measure of the influence of productivity on diversity (64). They demonstrated a unimodal correlation between diversity (observed as mutants) and productivity in heterogeneous, but not in homogeneous environments. At low substrate concentration, there were too few cells to create and inhabit specialized niches. Higher substrate levels and therefore higher population density, allowed evolution and selection for specialization to occur with the result of increasing diversity. Finally, at highest concentration the superior competitor excels, reducing the range of species.

A different relationship between biomass and diversity was observed during the build-up of the biofilm in our model drinking water distribution system. A reduction of diversity was observed during the period of growth, resulting in a negative correlation between activity and richness (paper 4). Why this discrepancy between those two systems? In my own system, a constant immigration of cells occurs from the bulk water creating bigger chances for colonization to happen. This recruitment will provide new species independently of productivity. Therefore, the diversity does not need to be generated in the community and will eliminate the reduction of diversity at low productivity. As a result, a high richness will be observed at low productivity. Furthermore, both studies describe a negative effect of high productivity on the apparent diversity in a heterogeneous system. The comparison describes how a complex interaction between the biofilm and adjacent bulk water population may influence the structure of the overall community.

Succession

The concept of successional theory provides the theoretical basis for the development of a community over time. Unfortunately, there is no generally accepted definition of succession, which is related to the discussion of what promotes successional changes. The pioneering and holistic concept view succession as a number of seral stages in which the community develops into a climax population. Each step either prepares (pioneering concept) or facilitates (holistic) the next and thereby through self-induced changes – autogenic changes – reaches an optimal community (Table 5). The climax community is characterized by a relative high diversity of K-specialists, low biomass production to respiration ratio, and complex food chains in comparison to earlier seral stages (108). In opposition to this view of an orderly process is the reductionists concept in which the individual members compete as well as adjust to environmental gradients and through these processes does the community develop. The competition can include facilitation by excreting compounds utilized by other organisms (producing new resources), inhibition by production of antagonistic compounds or tolerance by allowing new niches (e.g. anaerobic zones). Commonly for both theories is that successional processes overcome effects of differences in topography, localized gradients etc. with the effect that a population develops into a uniform community.

	Pioneering concept	Holistic concept	Reductionist concept
Climax	Developing into stable "superorganism"	Stable climax community Inhabited by k-specialist High diversity	Most competitive individuals
Driving force	Autogenic Each step prepare for replacement Orderly	Autogenic Self-induced modication driven Orderly	Allogenic Reacting to environmental gradients Competition/unpredictable

Table 5. Theory of succession

In terms of colonization of a surface and formation of a biofilm, succession could be viewed as a stochastic primary attachment of a number of species selected from the bulk water population. The process might not be entirely random, since certain bacterial species or strains have a greater aptitude for colonization (2, 126). Since the bacteria initially are spatially separated and have a low selection pressure in the ability of attachment, it most likely that a community with high diversity occurs. This initial attachment (facilitation) can be followed by a secondary colonization of bacteria that get a protective environment in the biofilm and/or feed of remnants of other bacteria, as well as the exclusion of less competitive organisms. The bacterial assemblage may simplify, as the superior competitor starts to dominate the community. This will result in a drop in diversity. As the bacterial biofilm matures, more niches are created due to formation of gradients and internal recycling of resources. At this seral stage richness and evenness increases, reflecting a complex spatial structure with many functional groups of bacteria (58) (paper 4).
So has microbial succession been observed in microbial biofilms? In a study by von Canstein and co-workers, where they looked at succession in a mercury-reducing biofilm, they observed how the richness followed a similar trend as described. Importantly, most of the early colonizers were still present in the late samples, showing that little exclusion occurred (168).

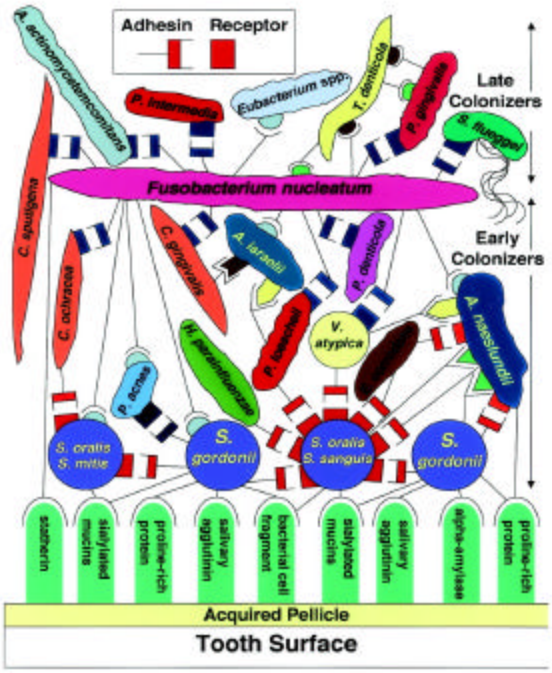


Figure 8. Spatiotemporal model of oral bacterial colonization (67)

A well-studied example is the colonization of dental surfaces, whereby the formation of a biofilm (dental plague) occurs through an orderly sequence of events (see Figure 8). The early colonizers incl. *S. gordonii* recognize surface molecules which act as receptors for attachment. Other bacteria identify surface receptors on the early colonizers and settle through coadhesion or coaggregation. *Fusobacterium nucleatum* act as principal coaggregation bridge between early and late colonizers, enabling a wide variety of species to attach to the biofilm (67). It is unclear if this succession is matched by a similar change in richness as proposed in the previous paragraph.

The analysis of factors influencing biofilm formation shows that the combination of traits of individual bacteria, the external physio-chemical environment, and the overall ecosystem functioning influences the composition of the biofilm.

Potential nutrients in drinking water

The specific chemical structure of the energy and carbon source is still unknown in drinking water and is probably of a very complex and varying composition. Humic acids and polysaccharides are proposed as common constituents of the total organic carbon (TOC), but the composition of the organic carbon is not known. Assimilable organic carbon (AOC) is the portion of total organic carbon that can be readily utilized by bacteria to support growth, and usually constitutes between 0.1 – 10% of the organic carbon in aquatic environments (163). This fraction of the total carbon is low molecular weight compounds including polysaccharides and phenolic compounds (50, 175).

AOC is measured as a bioassay in which the sample is inoculated with *P. fluorescens* P17 and (*Aqua*)*spirillum* NOX and maximum cell density is recorded. When correlated to a known yield coefficient, the AOC content can be estimated (163). The assay has been applied extensively to monitor "biological stability" of drinking water and some correlation to regrowth is observed (80, 82). It appears, that a *Pseudomonas* is a good choice as indicator organism due to a metabolic versatility, fast response and growth rate, and common presence in drinking water (paper 2). The readily utilized organic carbon probably includes most of the carbon used by bacteria in the bulk water phase, due to a retention time of less than 3 days in most distribution system. However in the biofilm, bacteria have sufficient time to degrade recalcitrant compounds and may therefore utilize other organic compounds than the bulk water population.

In certain systems, non-carbon compounds supported growth. Many drinking water utilities have implemented chloramination as disinfection residual in the distribution system. Chloramine is less reactive than chlorine and generates fewer disinfection by-products like trihalomethanes. However, chloramine act as substrate through oxidation to nitrite and nitrate and lead to a removal of disinfectant residual as well as biomass accumulation (124, 183). Even in the absence of chloramine, a high abundance of nitrifying bacteria can be observed in water supply systems, supporting the concept of nitrogen compounds as an important alternative energy source in drinking water (86) (paper 3).

Finally, phosphorous has been described as a limiting factor for growth in drinking water in certain boreal regions, due to a high concentration of carbon (85, 99). All these studies illustrate that other substrates than carbon may influence the potential for bacterial growth in drinking water.

Biofilm vs. bulk water populations in drinking water

There are several factors that differentiate the biofilm from bulk water population in drinking water: (i) higher abundance of grazing organisms (selecting for resistant strains), (ii) competition for nutrients combined with low productivity (growth), and (iii) selection based on attachment properties including production of extracellular polymeric substances (EPS) in order to avoid detachment due to shear force.

Grazing activity can be higher in biofilm compared to the bulk water, and may select for resistant strains. Sibille and co-workers showed how *E. coli* cells more readily colonized a biofilm in the absence of grazers (138). The higher concentration of protozoans in the biofilm may also affect the presence of *Legionella*, since this strain needs a host for multiplication.

The biofilms formed in these systems are usually less than 20 µm in thickness with bacterial cells loosely dispersed (paper 1 & 4). It is unknown if this biofilm morphology will affect the species composition, but inferred from the difference between 14-days-old and 1093-days-old biofilm in community composition, nutrients gradients may occur. The formation of gradients may create ecological niches for a variety of species to proliferate including anaerobic zones. Niches, those are not present in the bulk water. An additional mechanism is the adsorption of polymeric compounds like humic acids to the biofilm surface (40). This may provide the biofilm community with an opportunity to produce extracellular enzymes degrading recalcitrant compounds (40), as well as metabolic interaction (105). Among heterotrophic organisms, the most abundant surface associated strains in two investigated distribution systems were able to grow on a larger number of nutrients compared to strains isolated from the bulk water (paper 2). Strains that are able to grow on/uptake many substrates may be more competitive for resources since they have more transporter proteins in use, resulting in a higher growth rate (17). This result indicates a stronger competition for nutrients and could be explain by the higher cell density in the biofilm compared to the bulk water.

Finally, bacteria organized in a biofilm structure are known to produce EPS and other cell structures affecting attachment and structural organization. Due to the relative high flow rate observed in distribution systems, a selection of strains that adhere strongly to the surface may occur.

The bulk water is in contact with many environments during distribution, e.g. raw water source, filtering unit at water work, different pipe materials, gaskets etc. The bacterial content may therefore reflect the "history" of the water, rather than a community specialized for this habitat. The relatively even diversity pattern observed in the bulk water (paper 2 & 3) could be maintained through several processes: (i) resource saturation (enough resources leading to no competition and high diversity), (ii) resource fluctuation (non-equilibrium conditions and specialization resulting in high diversity), (iii) spatial isolation, and (iv) detachment of cells from the biofilm.

The nutrient content of the bulk water has a very low amount of biodegradable organic matter. An analysis of the growth potential (AOC measurement) shows that the nutrient concentration hardly can support the density of cells observed at present. However, the chemical composition changes radically after treatment at the waterwork (in Denmark from an anaerobic to an aerobic environment). As a result, the microbial composition in the bulk water may not be in equilibrium with the nutrient content of the water. Combined with resource fluctuations, this would possibly invoke more diversity in the water column.

The low density of cells in the bulk water results in an average distance between the bacteria of more than 100 µm. This results in a spatial isolation of the cells and makes room for many different species to proliferate and adapt independently (186). Nevertheless, the low concentration of cells will impede nutrient gradients to form in the water phase. This should create a uniform diversity in the bulk water.

The observed diversity in the bulk water is probably a consequence of a low density and activity of the bacteria present. The separation and low activity reduces competition and allows bacteria proliferation individually. Initially, the diversity is introduced by detaching cells from adjacent biofilm communities or biofilter at the waterwork. As a result, the bulk water reflects the "history of the water".

In the analysis of the model distribution system, it was observed that the bulk water had a higher richness than the attached community until the biofilm reached app. 2 years of age (paper 4). Whereas the bulk water can maintain a relative high diversity in the absence of growth and competition, the build-up of gradients in the biofilm will slowly generate a diverse environment and eventually a higher diversity in comparison to the bulk water.

The comparison illustrates how bacteria proliferate in two profoundly different environments, and this will influence the total population in a drinking water distribution system. Therefore, in order to examine the microbial diversity in this environment, it is necessary to include both the biofilm and the bulk water.

Concluding remarks

It is becoming clear, that despite our eradication of many pathogenic strains in potable water, other unwanted and potentially harmful bacteria are being recognized as present in the water supply systems in the absence of any clear correlation to a contamination event. It is impossible to completely eradicate bacteria from practically any possible system including drinking water networks, and harmful bacteria will be introduced occasionally. So the real task is to control the survival and proliferation of bacteria in the distribution network. It has been illustrated in the previous sections, that the fate of pathogens in the supply system is a result of a complex interaction between the cell and the local environment, and as an effect of this, the indigenous microbial population.

The aim of the present thesis is to determine the identity and structural organization of the bacteria present in a drinking water distribution system. The following four papers will demonstrate how previously unrecognized bacterial species in drinking water proliferate in two separate ecological compartments, in a biofilm on the pipe surface and as free-swimming in the bulk water.

Paper 1 shows how a biofilm formed in a simplified flow-cell system consisted of a mixed community containing a variety species at different trophic levels.

Paper 2 continue with describing the disparity between the population in the biofilm and the bulk water. In two geographically separated distribution systems, the easily culturable subpopulations were characterized phylogenetically and physiologically. The investigation showed how many of the bacterial groups found in the two systems were affiliated to known microorganisms found in drinking water. A carbon source utilization assay suggested, that despite phylogenetic differences between the two analyzed system, the bacterial response to a variety of substrates were clearly correlated to the subenvironment of isolation (biofilm vs. bulk), demonstrating a physiological adaptation of the microbial population.

As discussed in the introduction, a number of examinations of bacterial communities have shown how plate-counting and cultivation underestimate and bias the enumeration of cells present in a given environment (142), including drinking water distribution systems (62). In order to circumvent this bias, molecular techniques were employed to reveal aspects of the bacterial community composition in drinking water that were lost during plating and isolation and compare it to the previous analysis of isolated strains (paper 3). This analysis of the total population revealed, how the majority of the bacteria present never previously had been detected in a water distribution network. The dominance of *Nitrospira* suggested that nitrite is an important energy source in the analyzed system. The investigation added evidence to the differentiation of the microbial community between the bulk water and biofilm, even though the interaction between these two compartments appears complex.

Finally, paper 4 describes a successional pattern in the development of a biofilm in the drinking water distribution system. It appears, that not only does the population differentiate spatially between the water phase and the biofilm, but also temporally.

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